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Multicenter Evaluation of a Sequence-Based Protocol for Subtyping Shiga Toxins and Standardizing Stx Nomenclature

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When Shiga toxin-producing *Escherichia coli* (STEC) strains emerged as agents of human disease, two types of toxin were identified: Shiga toxin type 1 (Stx1) (almost identical to Shiga toxin produced by *Shigella dysenteriae* type 1) and the immunologically distinct type 2 (Stx2). Subsequently, numerous STEC strains have been characterized that express toxins with variations in amino acid sequence, some of which confer unique biological properties. These variants were grouped within the Stx1 or Stx2 type and often assigned names to indicate that they were not identical in sequence or phenotype to the main Stx1 or Stx2 type. A lack of specificity or consistency in toxin nomenclature has led to much confusion in the characterization of STEC strains. Because serious outcomes of infection have been attributed to certain Stx subtypes and less so with others, we sought to better define the toxin subtypes within the main Stx1 and Stx2 types. We compared the levels of relatedness of 285 valid sequence variants of Stx1 and Stx2 and identified common sequences characteristic of each of three Stx/Stx1 and seven Stx2 subtypes. A novel, simple PCR subtyping method was developed, independently tested on a battery of 48 prototypic STEC strains, and improved at six clinical and research centers to test the reproducibility, sensitivity, and specificity of the PCR. Using a consistent schema for nomenclature of the Stx toxins and *stx* genes by phylogenetic sequence-based relatedness of the holotoxin proteins, we developed a typing approach that should obviate the need to bioassay each newly described toxin and that predicts important biological characteristics.

Since the first discovery of Vero cytotoxin in 1977 (30), numerous Shiga toxins have been characterized, and the diversity of this toxin family has become clear. The study of cytotoxin-producing *Escherichia coli* simultaneously by several investigators around the globe resulted in the use of two different names, Vero cytotoxins (VT) and Shiga-like toxins (SLT), for the toxins produced by these bacteria. In 1994, O'Brien et al. (42) proposed that the nomenclature for the *E. coli* cytotoxins (SLT and VT) be considered interchangeable. Two years later, Calderwood et al. (12) suggested that "like" be omitted and the toxins and gene names reflect their relationship to Shiga toxin (Stx) from *Shigella dysenteriae* type 1, the prototype toxin for the family. To be inclusive of already-published reports, it was suggested that cross references to existing VT nomenclature be used. While the omission of the word "like" was readily accepted by the scientific community, strong arguments for maintaining the Vero cell phenotype nomenclature for *E. coli* cytotoxins were immediately put forward (27); thus, both systems of nomenclature are still being widely used. For consistency, the Stx nomenclature will be used throughout this report.

The Stxs share the following properties: operon structure (*stxA* immediately upstream of *stxB* with a short intergenic sequence); polypeptide subunit structure (five B subunits to one A subunit in the mature holotoxin); enzymatic activity (N-glycosidases); binding to specific glycolipid receptors; and biological properties, including enterotoxicity in ligated rabbit ileal loops, neurotoxicity in mice, and cytotoxicity to receptor-expressing tissue culture cell lines such as Vero and HeLa cells. The Shiga toxin family can be categorized into one of two branches, Stx1 (almost identical to Stx from *S. dysenteriae* type 1) and Stx2, because polyclonal antisera raised against one type does not neutralize toxins of the heterologous type. Correspondingly, Stx1 and 2 genes do not display DNA-DNA cross hybridization under conditions of high stringency. As new toxins were studied, the need for subtyping evolved. Some toxins were defined simply as Stx1 or Stx2 despite sequence and biological differences from the prototypic Stx1 and 2, while other toxins that differ from the prototypes in either group received arbitrary subtype designations from their discoverers. Subtypes, denoted by Arabic letters that follow the main type name,

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share cross hybridization of their genes under high stringency but may exhibit significant differences in biological activity, including serologic reactivity, receptor binding, and the capacity to be activated by elastase in intestinal mucus. The lack of uniform guidelines for defining and naming subtypes and the significant diversity among sequences within the main families have caused much confusion. It has been recommended that Shiga toxin family members be classified based on phenotypic differences, biological activity, and hybridization properties (42); however, not all variants have been examined for all these properties. Nonetheless, these toxin attributes are clinically relevant in that some subtypes or variants of Stx2 seem to be highly associated with serious sequelae, namely, the hemolytic uremic syndrome (HUS) (10, 16, 50). Other subtypes or variants of Stx1 and Stx2 are primarily associated with a milder course of disease (10, 16, 50), and Stx2e-producing Shiga toxin-producing *E. coli* (STEC) strains are probably not human pathogens (56). Consistent nomenclature and subtyping strategies are essential for surveillance and for predicting the risks associated with particular STEC infections.

A plethora of sequences have been examined and submitted to the Entrez Nucleotide database, a collection of sequences from several sources, including GenBank, RefSeq, and the PDB (Protein Data Bank), hosted by the National Center for Biotechnology Information (NCBI). However, very few of these many variants have been examined for all the classical phenotypic differences, biological activity, and hybridization properties. Several studies (2, 3, 13, 23, 32, 51) have described the phylogenetic relationship between some of these variants, but no study has thoroughly examined all variants. Here we compared *stx* sequences and grouped them according to genetic relatedness. Based on those results, we established a protocol for the subtyping of both stx_1 and stx_2 using PCR and tested it against a panel of 62 STEC reference strains especially established for this study, a small selection of 162 clinical isolates, and all 42 strains from the German HUSEC collection (35). A subset of the available reference strains was also tested for their capacity to be activated by mucus treatment. The nomenclature proposed and the PCR methodology tested both expand upon previous attempts (54, 58) to create a universal nomenclature for the Shiga toxins and preserve the subtype designations that are based on differences in biological properties of the toxins and predictable by sequence.

MATERIALS AND METHODS

Sequence analysis. We searched the scientific literature for Shiga, Vero cytotoxin, and Shiga-like toxin references that reported new toxin types and toxin sequences and the GenBank for *stx*-related sequences. Nucleotide sequences of the full *stx* operon that encode the signal peptides (66 bp in the A and 60 and 57 bp, respectively, in the B subunits of Stx/Stx1 and Stx2), the A subunit (879 bp in Stx/Stx1 and 891 bp in Stx2), the intergenic region (9-12 bp), and the B subunit (207 bp for *stx* and stx_1 and 204 or 210 bp for stx_2), as well as the amino acid (aa) sequences for the combined A and B holotoxin, were downloaded or translated from the open reading frames predicted by sequences to encode the holotoxin A and B subunit sequences of 404 aa for Stx/Stx1 and 406 aa for Stx2b (6 sequences), Stx2e, and Stx2f or 408 aa for Stx2a, Stx2b (10 sequences), Stx2c, Stx2d, or Stx2g and imported them into a BioNumerics (Applied Maths, Ghent, Belgium) database.

The holotoxin amino acid sequences of Stx/Stx1 and Stx2 were analyzed separately and compared by the unweighted pair group method using arithmetic averages (UPGMA), with an open gap penalty of 100%, a unit gap penalty of 0%, the fast algorithm at a minimum match sequence of 2, and a maximum number of gaps of 10, followed by multiple alignments and the creation of a consensus sequence from the root of the obtained dendrogram. Neighbor-joining cluster analysis with the same algorithm as that for UPGMA was used to analyze the global cluster calculations. Evolutionary unrooted trees were created from maximum parsimony cluster analysis using 100 bootstrap simulations. In addition, the amino acid sequences were analyzed for sequence motifs that would support the phylogenetic analyses.

The full nucleotide sequences, including the intergenic region, were analyzed by the same procedure to evaluate the possible differences between nucleotide and amino acid sequences. Discrepancies between the neighbor-joining and the maximum parsimony cluster analysis of the amino acid sequences were resolved using the evolutionary unrooted tree from maximum parsimony and compared to nucleotide analyses in order to assign subtypes and variants. The sequence for *S. dysenteriae 1* strain 3818T [accession no. M19437 (61)] was used as the reference sequence for the analysis of Stx/Stx1. The sequence for O157:H7 strain EDL933 [accession no. X07865 (24)] was used as the reference sequence for the analysis of Stx2. Partial sequences were excluded from the analyses in the assignment of variant designations. Sequences containing wobble bases were considered as valid and included if they represented synonymous substitutions and excluded as invalid when they represented nonsynonymous substitutions. A variant was defined by one amino acid difference in the analyzed sequences compared to the other sequences. The first valid published sequence was chosen to represent each specific variant. Cut-off values for subtypes were 95.89% similarity for Stx/Stx1 and 82.93% similarity for Stx2 except for the cluster of sequences for Stx2a, Stx2c, and Stx2d, which were analyzed separately because these subtypes are very closely related. Use of the above values for similarity would group them into one subtype; therefore, existing subtype designations were retained to highlight the significant differences in biological activities and virulence potential among these types and to avoid the introduction of additional confusion to the nomenclature of these cytotoxins.

Establishment of reference collection. The first and/or corresponding author(s) of the papers and online submissions of Stx reference sequences were contacted to establish a reference collection of strains. Submitted strains were O:K:H serotyped using conventional phenotypic antisera (46, 55) and analyzed for flagellar *fliC* genotypes by PCR and restriction fragment length polymorphisms (RFLP) of HhaI-digested *fliC* products (6). Biochemical characterization of the strains was determined according to the methods of Kauffmann (28). The production of Shiga toxin was detected by the Vero cell assay (VCA) (53) and with a commercial enzyme immunoassay [Ridascreen enzyme immunoassay (EIA); R-Biopharm AG, Darmstadt, Germany] (8). The strains were examined for the presence of virulence genes by dot blot hybridization using DNA probes NTP705, Shiga toxin 1 (stx_1) (64), DEP28, Shiga toxin 2 (all variants of stx_2 except stx_{2f}) (62), and a 625-bp PCR fragment amplified from the Statens Serum Institut (SSI) clinical isolate C 548-06, serotype O145:H34 using primers F4f/R1-ef (50) to detect stx_{2f}. All strains were then tested by the subtyping protocol described in this paper and with *stx*-specific PCR followed by RFLP analysis of PCR products as described previously (7).

Partial sequencing. Partial sequencing was used to verify toxin sequences from the reference collection strains. All variants of stx/stx_1 were sequenced with primers stx_1-seq-F1/stx_1-seq-R1 (this study) (Table 1) and of stx_2 with primers F4/R1 and F4-f/R1-e/f as previously described (50) on an ABI3130xl (Applied Biosystems) sequencer using a POP7 polymer (Applied Biosystems; catalog no. 4363785) and BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems; catalog no. 4337450) with minor modifications. Well-defined single colonies were inoculated in beef broth and incubated overnight at 37°C. One hundred microliters of broth was added to 900 μl of sterile H_2O, placed in a heating block at 100°C for 15 min, and centrifuged at 18,000 × *g* for 5 min. Upon transfer to a clean tube, the supernatant was used directly for PCR and stored at −18°C for further analyses. PCR was done in a total volume of 20 μl with 2.5 μl H_2O,

TABLE 1 Primers developed in this study, except primers for sequencing and detection of stx_2 (50)[a]

Gene(s), primer use, and primer	Sequence (5′–3′)[b]	Position	Amplicon size (bp)	Comments
stx and *stx1*				
Sequencing				
stx1-seq-F1	ATGTCATTCGCTCTGCAATAGGTAC	119–143	1,020	
stx1-seq-R1	GAAGAAGAGACTGAAGATTCCATCTG	1,113–1,138		
Detection				
stx1-det-F1	GTACGGGGATGCAGATAAATCGC	440–462	209	
stx1-det-R1	AGCAGTCATTACATAAGAACG**Y**CCACT	622–648		
Subtyping				
stx1a-F1	CCTTTCCAGGTACAACAGCGGTT	362–384	478	All 6 primers can be used in a triplex PCR for subtyping of stx/stx_1[c]
stx1a-R2	GGAAACTCATCAGATGCCATTCTGG	815–839		
stx1c-F1	CCTTTCCTGGTACAACTGCGGTT	362–384	252	
stx1c-R1	CAAGTGTTGTACGAAATCCCCTCTGA	588–613		
stx1d-F1	CAGTTAATGCGATTGCTAAGGAGTTTACC	50–78	203	
stx1d-R2	CTCTTCCTCTGGTTCTAACCCCATGATA	225–252		
stx_2				
Sequencing and detection				
F4	GGCACTGTCTGAAACTGCTCCTGT	606–629	627	For detection, all 4 primers can be used in one reaction; for sequencing, use F4 and R1 for all subtypes except stx_{2e} and stx_{2f}, which are sequenced with F4-f and R1-e/f
R1	ATTAAACTGCACTTCAGCAAATCC	1,209–1,232		
F4-f	CGCTGTCTGAGGCATCTCCGCT	606–629	625	
R1-e/f	TAAACTTCACCTGGGCAAAGCC	1,209–1,230		
Subtyping				
stx2a-F2	GCGATACTG**R**G**B**ACTGTGGCC	754–774		
stx2a-R3	CCG**K**CAACCTTCACTGTAAATGTG	1,079–1,102	349	
stx2a-R2	GCCACCTTCACTGTGAATGTG	1,079–1,100	347	
stx2b-F1	AAATATGAAGAAGATATTTGTAGCGGC	968–994	251	
stx2b-R1	CAGCAAATCCTGAACCTGACG	1,198–1,218		
stx2c-F1	GAAAGTCACAGTTTTTATATACAACGGGTA	926–955	177	
stx2c-R2	CCGGCCAC**Y**TTTACTGTGAATGTA	1,079–1,102		
stx2d-F1	AAARTCACAGTCTTTATATACAACGGGTG	927–955		
stx2d-R1	TT**Y**CCGGCCACTTTTACTGTG	1,085–1,105	179[d]	
stx2d-O55-R	TCAACCGAGCACTTTGCAGTAG	1,140–1,161	235	
stx2d-R2	GCCTGATGCACAGGTACTGGAC	1,184–1,206	280	
stx2e-F1	CGGAGTATCGGGGAGAGGC	695–713	411	
stx2e-R2	CTTCCTGACACCTTCACAGTAAAGGT	1,080–1,105		
stx2f-F1	TGGGCGTCATTCACTGGTTG	451–475	424	
stx2f-R1	TAATGGCCGCCCTGTCTCC	856–874		
stx2g-F1	CACCGGGTAGTTATATTTCTGTGGATATC	203–231	573	
stx2g-R1	GATGGCAATTCAGAATAACCGCT	771–793		

[a] PCR conditions are as described in the text below, except annealing temperatures, which were 56°C for sequencing and detection and 64°C to 66°C for the subtyping of stx/stx_1 or stx_2. Especially, the resolution of stx_{2a}, stx_{2c}, and stx_{2d} may require individual calibration of thermocyclers. A well-defined single colony is inoculated in beef broth and incubated overnight at 37°C. One hundred microliters of broth is added to 900 μl of sterile H_2O, placed in a heating block at 100°C for 15 min, and centrifuged at 18,000 × *g* for 5 min. Upon transfer to a clean tube, the supernatant is used directly for PCR and stored at −18°C for further analyses. For PCR, a total volume of 20 μl contains 2.5 μl H_2O, 10 μl HotStarTaq Master Mix Kit (Qiagen), 1.25 μl of each of two primers (stock solution of primers is 5 μM) and 5 μl supernatant of boiled lysate (stock). The thermocycler conditions are 95°C for 15 min followed by 35 cycles of 94°C for 50 s, 56°C for sequencing and detection, and 64°C for subtyping for 40 s and 72°C for 60 s, ending with 72°C for 3 min. PCR amplicons are stored at 4°C. The final resolution of stx_{2a}, stx_{2c}, and stx_{2d} may require calibration of individual brands of thermocyclers by testing annealing temperatures from 64°C to 66°C on the test panel of reference strains. In our hands, an additional PCR using the stx_{2d} primers was run at an annealing temperature of 66°C. False-positive stx_{2c} fragments disappeared and true stx_{2d}-positive fragments persisted at this annealing temperature. A total volume (20 μl) for standard PCR contains 2.5 μl H_2O [if three primers are used (stx_{2a}), the H_2O volume is reduced to 1.25 μl; if four primers are used (stx_{2d} or detection of all stx_2 variants), H_2O is not added]; 10 μl Mastermix (HotStarTaq, Qiagen); 1.25 μl of each of two primers (stock solution of primers is 5 μM) [if three primers are used (stx_{2a}), the H_2O volume is reduced to 1.25 μl; if four primers are used (stx_{2d} or detection of all stx_2 variants), H_2O is not added]; and 5 μl supernatant of boiled lysate (stock).

[b] Wobble bases are shown in bold.

[c] For triplex PCR for subtyping of stx_1, a total volume of 25 μl contains 12 μl Mastermix (HotStarTaq, Qiagen), 1 μl of each of the four primers for stx_{1c} and stx_{1d} (stock solution of primers is 5 μM), 2 μl of each of two primers for stx_{1a} (stock solution of primers is 5 μM), and 5 μl supernatant of boiled lysate (stock).

[d] All three reverse primers in the same reaction will result in amplicons of 179 bp with nine stx_{2d} variants, 235 bp with variant stx_{2d}-O55-5905, 280 bp with five stx_{2d} variants, and finally two amplicons of 179 bp and 280 bp with variant stx_{2d}-O73-C165-02.

10 μl HotstarTaq Master Mix Kit (Qiagen), 1.25 μl each of two primers (5 μM), and 5 μl supernatant of boiled lysate. The thermocycler conditions were 95°C for 15 min followed by 35 cycles of 94°C for 50 s, 56°C for 40 s, and 72°C for 60 s, ending with 72°C for 3 min. PCR amplicons were stored at 4°C.

PCR using primers stx_1-seq-F1/stx_1-seq-R1 generated a 1,020-bp (positions 119 to 1138) internal fragment of stx_1, within which the forward sequence GCAATAGGTAC and the reverse sequence AGATGGAAT were used as in-frame trimming sequences in BioNumerics comparisons. Upon removal of the intergenic region, the nucleotide sequences were translated into 326 amino acids, covering 272 residues of the C-terminal part of subunit A and 54 residues of the N-terminal part of subunit B (see Fig. S1 in the supplemental material). PCR using primers F4/R1 and F4-f/R1-e/f generated a 625-bp (positions 606 to 1230) or 627-bp (positions 606 to 1232) fragment of stx_2, of which the forward sequence GAAAC TGCT (or GAGGCATCT for stx_{2f}) and the reverse sequence GGATTTG (or GGCTTTG for stx_{2e} and stx_{2f}) were used as in-frame trimming sequences in BioNumerics comparisons. Upon removal of the intergenic region, the nucleotide sequences were translated into 200 amino acids, covering 114 residues of the C-terminal part of subunit A and 86 residues of the N-terminal part of subunit B (see Fig. S2 in the supplemental material). Amino acid sequences and nucleotide sequences were analyzed by comparison with the established reference sequences in BioNumerics using the same settings as mentioned above. Chromatograms with double peaks were assumed to contain two or three different toxin genes and were examined with the subtyping protocol described below.

Development and validation of subtyping protocol. Based on the sequence analysis, a list of reference amino acid sequence variants was established for both Stx/Stx1 and Stx2. The reference nucleotide sequences for variants of stx/stx_1 and stx_2 were then aligned and searched to identity-conserved areas within each subtype for the development of subtype-specific primers that could be used in a new protocol for the subtyping of stx/stx_1 and stx_2 by PCR. Care was taken to design primers that required similar PCR conditions, and only sequences within the holotoxin sequence were used. The tested primers and running conditions are listed in Table 1. Forty-eight (marked in bold in Table 2) of the 62 strains from the reference collection were sent without identification to the six centers along with the PCR protocol. Initially, an annealing temperature of 62°C was used for subtyping both stx_1 and stx_2. Laboratories that submitted false-positive results were asked to retest at an annealing temperature of 64°C to 66°C and/or to use the HotStarTaq Master Mix Kit (Qiagen) if another DNA polymerase had been used in the first test.

Test of subtyping protocol on clinical isolates. The 62 submitted reference strains, the German HUSEC collection (35), and 162 (primarily human) clinical isolates covering the years 1994, 1998, and 2000–2010 from Denmark (125 isolates), Belgium (27 isolates), and Germany (7 isolates) plus 3 representative isolates from Australia, New Zealand, and Switzerland (1 from each country) were tested by this subtyping protocol at the SSI, Copenhagen, Denmark. Previous data on the clinical strains tested indicated that 40 strains were positive for stx_1, 45 were positive for stx_2, 33 were positive for stx_{2f}, and 44 were positive for stx_1 and stx_2.

Activatability. A subset of 23 strains from the reference collection that contained a representative non-Stx2d single subtype or any of the observed combinations of Stx subtypes and including 11 of the 13 strains positive for Stx2d was tested for toxin activatability by treatment of culture lysates with mucus collected from mouse intestines as previously described (36). Vero cell cytotoxicities of the lysates following buffer or mucus treatment were compared.

RESULTS

Sequence analysis. A total of 398 sequences (85 stx/stx_1 and 311 stx_2 plus two not specified) were identified. Thirteen (2 stx_1 and 11 stx_2) sequences were invalid, and 100 were partial sequences (36 stx_1 and 64 stx_2) and were excluded from further analyses, resulting in a total of 285 valid (47 stx/stx_1 and 238 stx_2) sequences. The 398 sequences are listed by toxin type, subtype, and *stx* nucleotide variant designations for valid sequences (see Appendix SA in the supplemental material). The partial or invalid sequences are also included.

Stx/Stx1. Forty-seven Stx and Stx1 amino acid sequences fell into three clusters with 13 unique subtypes (Fig. 1). Two clusters corresponded to the existing subtype nomenclature for subtypes Stx1c (47, 66) and Stx1d (11, 44). In addition to variant Stx1c-O174-DG131-3 (47, 66), variants from Ount HI-N (2), ONT HI-A (1), and ONT HI-C (1) were identified as belonging to subtype Stx1c. Only variant Stx1d-ONT-MHI813 (11, 44) was found in the subtype Stx1d cluster. Two identical amino acid sequences were found for *S. dysenteriae* (strain 3818T) and *Shigella sonnei* (strain CB7888). One silent nucleotide substitution was present in the B subunit of *S. sonnei*, strain CB7888 [accession no. AJ132761 (9)]. The Stx sequence was present in a cluster that also included sequences from O157 strains EDL933 (25) and AI2001-52 (deposited into the Entrez Nucleotide database by M. Suzuki et al. in 2002 under accession number AB083044), O111 strains PH (47), CB168 (48), 3385-00 (66), and 04-06263 (67), and O48 strain 94C (47). This cluster was designated subtype Stx/Stx1a. Similarities ranged from 97 to 98.3% between Stx1a and Stx1c, from 95.4 to 95.9% between Stx1a and Stx1d, and from 95 to 96% between Stx1c and Stx1d. Similarities within Stx/Stx1a were 99.2 to 99.8% and within Stx1c were 98.3 to 99.6%.

Stx2. Ninety-three unique Stx2 sequences were identified among the 238 sequences. Thirty-five different amino acid sequences fell into four clearly defined clusters, while one sequence (accession no. AM904726) was significantly different yet had some similarity to these four clusters (see below and Fig. 2).

Three of these four clusters included sequences represented in the existing subtype nomenclature for subtypes Stx2e, Stx2f, and Stx2g. One cluster included 16 sequences represented by the subtype Stx2b as proposed by Persson et al. (50).

A large group comprised 57 individual sequences with a minimum similarity of 97.45% (range, 97.45 to 99.87%). This group included Stx2 from O157-EDL933, Stx2c from O157-E32511, and the two Stx2d toxins from O91-B2F1. Therefore, these subtypes were analyzed separately (Fig. 3). Neighbor-joining cluster analysis of 18 amino acid sequences, including the prototype Stx2c from the O157-E32511 sequence, formed a separate cluster, which was designated subtype Stx2c. Another 19 sequences, including the prototype Stx2 from O157-EDL933, also fell into one cluster, which we designated Stx2a. Twelve sequences, including both prototypes Stx2d1 and Stx2d2 found in strain B2F1, formed a third cluster, which was closely related to a small group of five sequences. Together these two clusters of 17 sequences were designated subtype Stx2d. An evolutionary tree derived by maximum parsimony confirmed these three clusters (Fig. 3).

Of the four sequences outside the Stx2a, Stx2c, and Stx2d clusters, two sequences, accession nos. EF441619 (32) and AY633459 (40), fell into the Stx2d cluster by neighbor-joining analysis of their amino acid sequences, but both parsimony of the amino acid sequences and neighbor-joining cluster analysis of the underlying nucleotide sequences indicated that these two sequences fell within the Stx2a cluster. These two variants were therefore designated Stx2a-O104-G5506 and Stx2a-O8-VTB178, respectively. This placement was further corroborated by motif analysis and PCR subtyping.

One amino acid sequence, accession no. DQ059012, previ-

TABLE 2 Summary of the results on the reference collection of two *Shigella* and 60 *E. coli* strains (9 Stx1 and 51 Stx2) submitted to the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* after serotyping and using the subtyping protocol (Table 1)

Strain or plasmid used for validation of the detection and subtyping protocol (reference)[a]	Strain serotyped as part of this study[b] (original published serotype)	Result(s) obtained using the protocol described in Table 1 and in the text	Result(s) obtained using PCR/RFLP *stx* subtyping[c]
3818T (61)	*S. dysenteriae*	*stx*	
CB7888 (9)	*S. sonnei* [H16]	*stx*	stx_1
EDL933 (43)	O157:H7	stx_{1a} and stx_{2a}	stx_1
H-19B (30)	O26:H11	stx_{1a}	stx_1
H30 (30)	O26:H11	stx_{1a}	stx_1
04-06263 (67)	O111:NM	stx_{1a}	
CB168 (47)	O111:[H8]	stx_{1a}	stx_1
PH (48)	O111:[H8 defective]	stx_{1a} and stx_{2a}	stx_1
94C (47)	O48:H21	stx_{1a} and stx_{2a}	stx_1
DG131/3 (29, 47)	O174:H8	stx_{1c} and stx_{2b}	stx_{1c}
MHI813 (11)	O8:K85ab:HR (ONT:H19)	stx_{1d}	stx_{1d}
258/98[d]	O157:[H7]	stx_{2a}	stx_2
126814 (4)	O26:H11	stx_{2a}	stx_2
VTB60 (40)	O165:H25 (O136:H-)	stx_{2a} and stx_{2c}	stx_2 and stx_{2v}-ha gene
A397 (40)	O157:H7	stx_{2a} and stx_{2c}	stx_2 and stx_{2v}-ha gene
93-111 (32)	O157:H7	stx_{1a} and stx_{2a}	
928/91 (32)	O111:H-	stx_{1a} and stx_{2a}	
EBC201 (13)	O101:[H10]	stx_{2a}	stx_2
pEHEC400[e] (13)	OR:H48	stx_{2a}	stx_2
EBC217 (13)	O22:H8 (NT)	stx_{1a} and stx_{2a}	stx_1 and stx_2
I6581 (32)	O157:H7	stx_{2a} and stx_{2c}	
G5506 (32)	O104:H21	stx_{2a}	
VTB178 (40)	O8:H19	stx_{2a}	stx_2
CL-3 (32)	O113:H21	stx_{2a} and stx_{2d}	
031 (49)	O174:H21	stx_{2b} and stx_{2c}	stx_{2b}
EH250 (51)	O118:H12	stx_{2b}	stx_{2b}
3143/97 (52)	O22:H8	stx_{1c} and stx_{2b}	stx_{1c} and stx_{2b}
5293/98 (65)	O40:H8	stx_{2b}	stx_{2b}
24196/97 (65)	O128:H2 (O86:H-)	stx_{2b}	stx_{2b}
E32511 (60)	O157:[H7]	stx_{2a} and stx_{2c}	stx_2 and stx_{2v}-ha gene
C394-03 (50)	O157:[H7 defective]	stx_{1a} and stx_{2c}	stx_{2v}-ha gene
469 (40)	O157:H7	stx_{2c}	
EBC287 (13)	O171:K49:H2 (NT)	stx_{2c} and stx_{2b} and stx_{2d}	stx_{2v}-ha gene and stx_{2v}-hb gene
310 (40)	O157:H7	stx_{2c}	stx_{2v}-ha gene
A75 (40)	O157:H7	stx_{1a} and stx_{2c}	stx_1 and stx_{2v}-hb gene
pVTEC9 (13)	OR:K+:H-	stx_{2c}	stx_{2v}-ha gene
EBC219 (13)	O?:K17:H7 (NT)	stx_{2c}	stx_{2v}-ha gene
06-5121 (20)	O177:[H25]	stx_{2c} and stx_{2d}	stx_{2v}-ha gene and stx_2-NV206
B2F1 (22)	O91:H21	stx_{2d}	stx_{2v}-ha gene
MT 71 (26)	O28ab:H9 (O28:H28)	stx_{2d}	
7279 (39)	O157:H7	stx_{2c}	stx_{2v}-ha gene
C466-01B (50)	O8:H19	stx_{2d}	stx_{2v}-hb gene
EC173b (21)	O174:H21	stx_{2b} and stx_{2d}	stx_{2v}-hb gene and stx_b
EC1720a (21)	O174:H21	stx_{2b} and stx_{2d}	stx_{2v}-ha gene and stx_{2b}
EC1871a (21)	O165:H11 (NT)	stx_{2d}	stx_{2v}-hb gene
EBC275 (13)	O2:H29 (NT)	stx_{2b} and stx_{2d}	stx_{2v}-ha gene
pVTEC7 (13)	OR:K+:H48	stx_{2d}	stx_{2v}-hb gene
C165-02 (50)	O73:H18	stx_{2d}	stx_{2d}-O73
NV206 (5)	O6:H10	stx_{2d}	stx_2-NV206
5905 (32)	O55:[H7]	stx_{2d}	
S1191 (63)	O139:K12:H1	stx_{2e}	
3615-99 (52)	O8:H10 (O22:H8)	stx_{2e}	stx_{2e}
E-D42 (15)	O101:H-	stx_{2e}	stx_{2e}
E-D43 (15)	O101:[H9] (O101:[H14])	stx_{2e}	stx_{2e}
E-D53 (15)	O101:[H9] (O101:[H14])	stx_{2e}	stx_{2e}
E-D68 (15)	O101:[H9] (O101:[H14])	stx_{2e}	stx_{2e}
2771 (41)	OR:H-	stx_{2e}	

(Continued on following page)

TABLE 2 (Continued)

Plasmid or strain used for validation of the detection and subtyping protocol (reference)[a]	Strain serotyped as part of this study[b](original published serotype)	Result(s) obtained using the protocol described in Table 1 and in the text	Result(s) obtained using PCR/RFLP *stx* subtyping[c]
FHI-1106-1092	O8:H2[f]	stx_{2a} and stx_{2d}	
T4/97 (59)	O128ac:[H2]	stx_{2f}	stx_{2f}
H.I.8. (18)	O89:[H2] (O128:H2)	stx_{2f}	stx_{2f}
7v (33)	O2:H25	stx_{2g}	stx_{2g}
S86 (19)	O2:H25	stx_{2g}	stx_{2g}

[a] The 48 strains selected for validation of detection and subtyping are shown in bold type. The availability of these strains is described in the supplemental material.
[b] Serotyping was done independently in two of the participating laboratories. Square brackets around an H type indicate genotypic *fliC* typing by PCR and RFLP analysis of nonmotile strains using the method described by Beutin et al. (6).
[c] Results obtained using the PCR/RFLP *stx* subtyping described by Beutin et al. (7).
[d] Deposited into the Entrez Nucleotide database by M. Bielaszewská et al. in 2002 under accession number AF524944.
[e] The original gene was cloned from wild-type strain EBC201 into plasmid pEHEC400 and published as such. Both EBC201 and pEHEC400 (with the cloned gene) were used in the validation of the subtyping protocol. We have resequenced and confirmed the stx_{2a} gene and serotyped the EBC201 wild-type strain.
[f] Serotype confirmed on a non-toxin-producing isolate by the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*.

ously designated Stx2d-O73-C165-02 (50), fell between the Stx2d and Stx2b clusters and well outside the subtype Stx2d cluster. By parsimony of the amino acid sequences and neighbor-joining cluster analysis of the underlying nucleotide sequences, this sequence was more similar to the Stx2d cluster (minimum similarity of 98.3% to all the *E. coli* Stx2d variants and 98.8% to the *Citrobacter freundii* variant) than to any of the variants in the Stx2b cluster (maximum similarity of 96.9%). Therefore, this variant was assigned to subtype Stx2d and kept as Stx2d-O73-C165-02. The last outlying sequence, accession no. AM904726, was distinctly different, with a minimum amino acid similarity of 96.7% to the Stx2e cluster and a maximum similarity of 97.6% to the Stx2d cluster. In contrast, the corresponding nucleotide sequence similarities were 90.6 and 98.6%, respectively, and as high as 97.7% to the Stx2a cluster. However, the evolutionary tree placed this sequence closer to that of the Stx2e cluster; therefore, we assigned it to the Stx2e cluster with the designation Stx2e-O8-FHI-1106-1092 (Fig. 2).

The intergenic regions between *stxA* and *stxB* fell into four groups of sizes from 9 to 12 nucleotides. The Stx1 intergenic regions were highly conserved with 9 nucleotides (GGGGGTAAA) between the A and B coding regions of Stx1a and Stx1d and 10 nucleotides (GGGGGGTAAA) in the Stx1c operon. The high similarity between Stx2a, Stx2c and Stx2d was also evident in their intergenic regions, which contained 11 nucleotides (AGGAGTTA AGC/T), in contrast to the 12 nucleotides found in the Stx2b (CA /T/GGGAGTTAAAT), Stx2e (AAGGAGTTAAGA), Stx2f (CAGG GGGTGAAT), and Stx2g (AAGGAGTTAAGC/T) operons (summarized in Table S1 in the supplemental material).

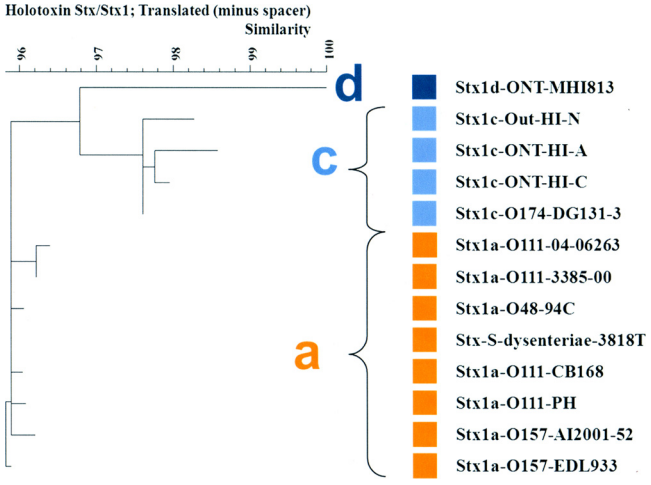

FIG 1 Three clusters of Stx and Stx1: neighbor-joining cluster analysis of 13 unique sequences as described in the text and the proposed new designations.

Common motifs. Common motifs were sought to support the phylogenetic analyses and to illustrate their association with biological activities such as the activatable property of Stx2d, as well as to assist in future assignments of subtypes. The alignment of all 13 Stx/Stx1 variants is shown in Fig. S1 in the supplemental material. The alignment of 93 Stx2 variants was grouped by subtype and characteristic common motifs as shown in Fig. S2 in the supplemental material. Particular interest was given to what has been referred to as "the activatable tail" (38) in Stx2d. This sequence consists of the last 10 amino acids in the C-terminal end of the A_2 subunit and has been identified as KSQSLYTTGE from position 288 to 297 (see Fig. S2). The two underlined amino acids, serine at position 291 and glutamic acid at the final position 297, have been indicated as recognition sites for the activation of the toxin by elastase, which results in a 35- to 350-fold increase in toxicity against Vero cells (36) and is associated with an extremely low oral 50% lethal dose of less than 10 bacteria when the bacteria that produce this toxin are fed to streptomycin-treated mice (34). This sequence is found in 2 stx_{2a} variants, all 4 variants of subtype Stx2g, 5 variants of Stx2e, and all 18 variants of Stx2d. As several subtypes, including the Stx2b-O118-EH250 variant and Stx2e, have been shown not to be activatable and at least one variant, Stx2d-O28-MT71, has been shown to be activatable (26), it is very likely that the B subunit plays an additional important role in determining whether the holotoxin is activatable; indeed, the B subunit of Stx2e was shown to prevent the activation of Stx2d in a chimeric toxin (37). One motif, END at position 14 to 16 in the B subunit, was found in all 18 variants of Stx2d, in subtype Stx2c, and in Stx2b. However, since neither Stx2b nor Stx2c contains the activatable tail, it seems that the combination of the activatable tail and the END motif in the B subunit together are responsible for the activatable property of the toxin (boxed in Fig. S2 in the supplemental material).

Establishment of a reference collection. A total of 2 *Shigella* and 60 *E. coli* strains (9 Stx1 and 51 Stx2), shown in Table 2, were

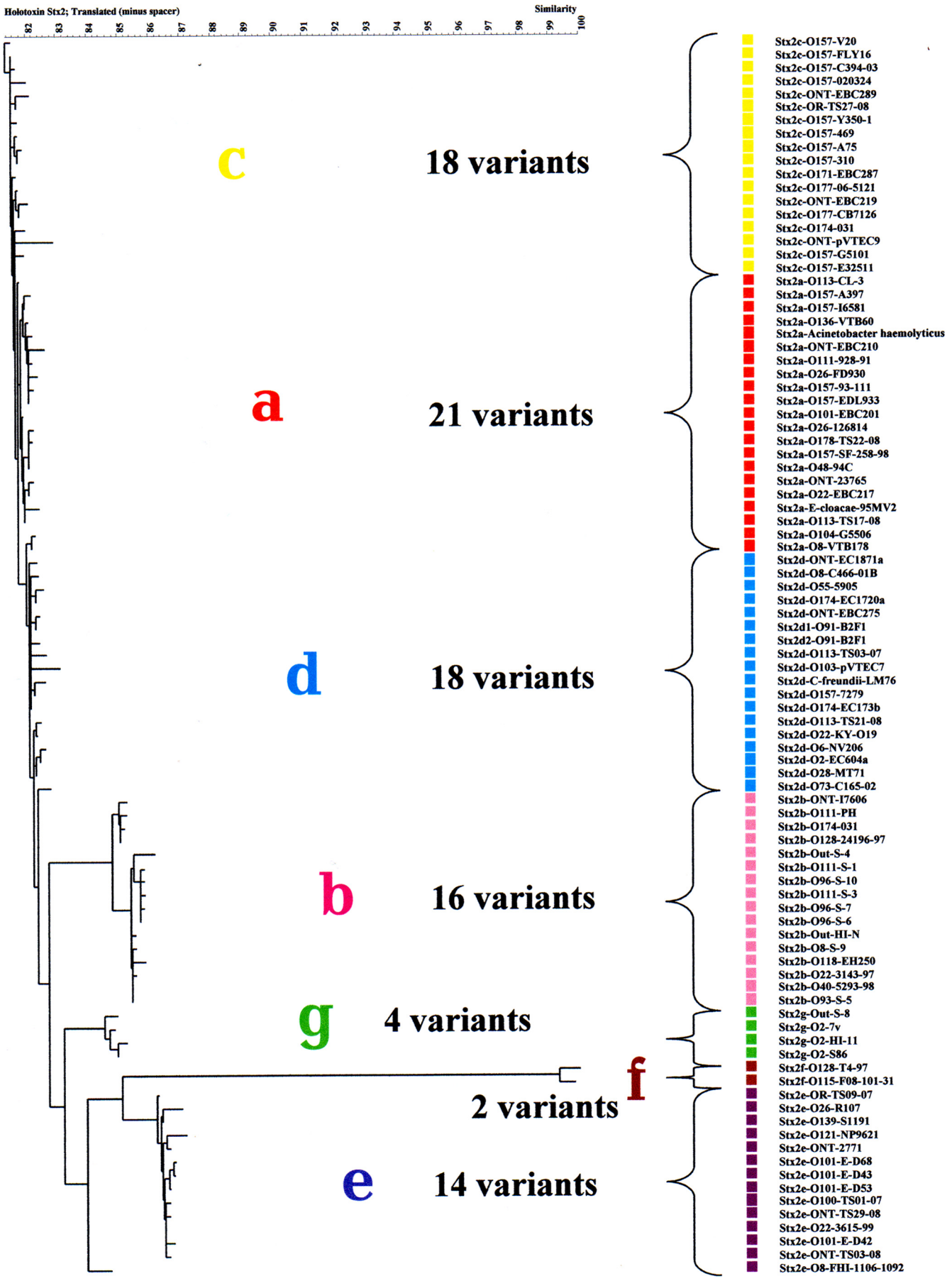

FIG 2 Five clusters of Stx2: neighbor-joining cluster analysis of 93 unique sequences as described in the text and the proposed new designations.

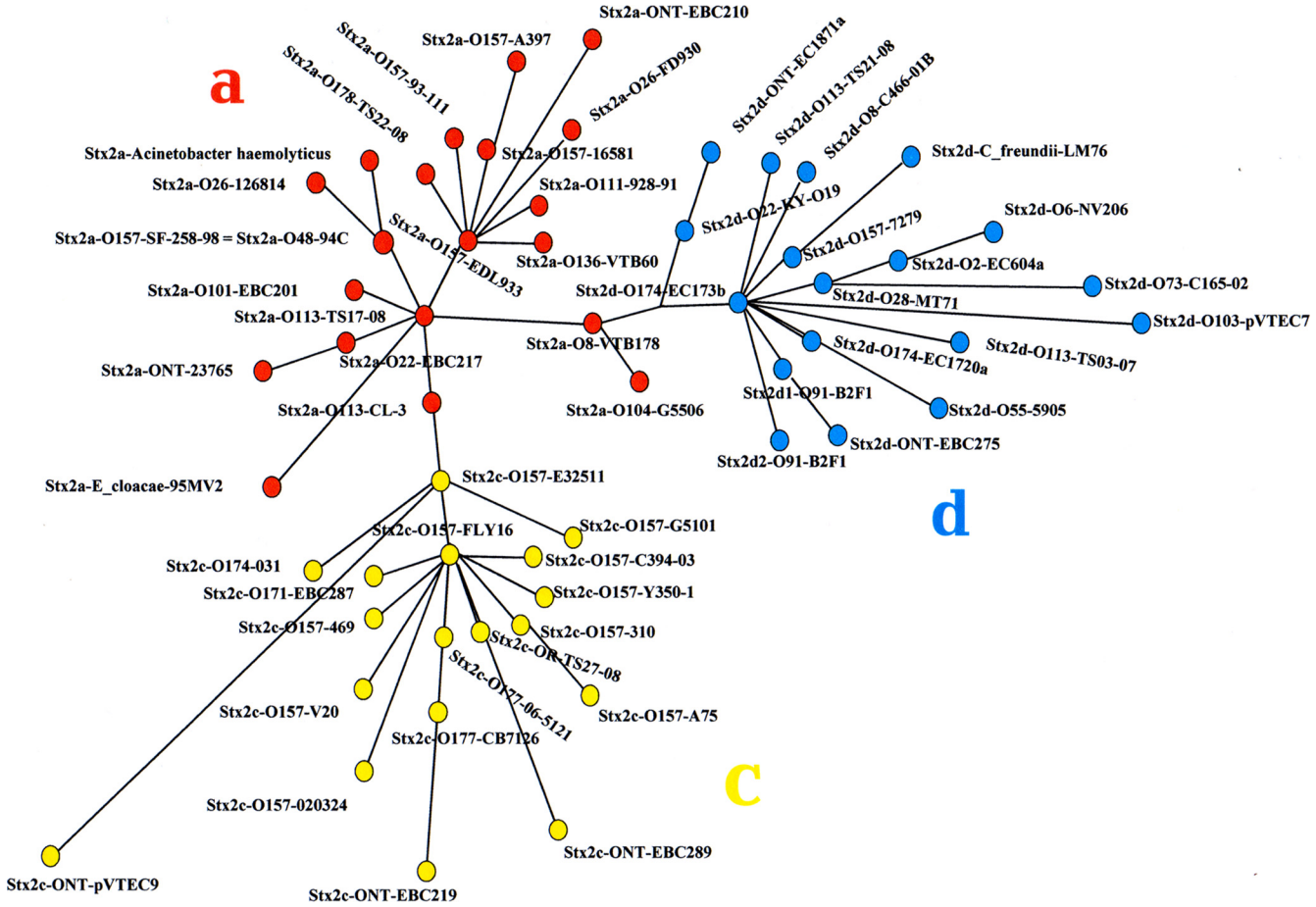

FIG 3 Maximum parsimony tree of 57 aa sequences: Stx2a, Stx2c, and Stx2d analyzed separately.

submitted to the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*. Using the subtyping protocol (Table 1) on the reference collection confirmed the presence of the expected *stx* genes in 47 strains and was in accordance with original publications by both partial sequencing and serotyping.

One strain, O157:H7 (7279), was negative by VCA, positive by Ridascreen EIA (8), positive for stx_2 by dot blot hybridization, negative with primers F4/R1 and F4-f/R1-e/f in four laboratories (and gave an odd-size fragment in two laboratories), and positive for stx_{2c} by the subtyping protocol and Stx2v-ha by the RFLP subtyping procedure (7). Three strains (24196/97, 3143/97, and 5293/98) were typed as stx_{2b} instead of the published stx_{2d}, and one strain (469) originally typed as stx_2 was retyped as stx_{2c}. Seven strains contained one more subtype than originally published: strain 06-5121 (stx_{2c}) was positive for stx_{2c} and stx_{2d}, A397 (stx_2) for stx_{2a} and stx_{2c}, A75 (stx_{2c}) for stx_{1a} and stx_{2c}, EBC217 (stx_{2a}) for stx_{1a} and stx_{2a}, EBC275 (stx_{2d}) for stx_{2b} and stx_{2d}, EC1720a (stx_{2d}) for stx_{2b} and stx_{2d}, and EC173b (stx_{2d}) for stx_{2b} and stx_{2d}. Only in one strain (PH) were we unable to detect the published subtype stx_{2b}, but we did detect both the stx_{1a} and stx_{2a} subtypes.

The O:H serotypes of 32 strains were confirmed. Seven nontyped (NT) strains were fully O:K:H serotyped for the first time, and 13 nonmotile or NT strains were H typed phenotypically (1 strain) or by molecular *fliC* typing (12 strains). In three strains, both the O group and the H type differed from those described in the original publication, and in one strain we failed to confirm the H type. Table 2 shows the summary of our results on the reference collection of the 62 strains after serotyping and using the subtyping protocol and on the 48 strains selected for validation of detection and subtyping. Table S2 in the supplemental material summarizes the proposed prototype, subtype, and variant designations of the Shiga toxins and genes as confirmed or updated by our studies along with the prototype strain name and any previous toxin designations that have been used to describe them.

Underlined in Table S2 (in the supplemental material) are 10 strains that we chose as reference strains for the three subtypes of Stx/Stx1 and seven subtypes of Stx2. They are *S. dysenteriae* strain 3818T (61) for Stx, *E. coli* strain EDL933 (43) for subtypes Stx1a and Stx2a, *E. coli* strain DG131/3 (29, 47) for subtype Stx1c, and *E. coli* strain MHI813 (11) for subtype Stx1d. *E. coli* strain 031 (49) produces two Stx2 subtypes, Stx2b and Stx2c. Strain EH250 (51) produces subtype Stx2b, and strain C165-02 (50) produces subtype Stx2d (for which two bands were amplified with our subtyping protocol). Strain S1191 (63) was used as the reference strain for subtype Stx2e, strain T4/97 (59) for subtype Stx2f, and strain 7v (33) for subtype Stx2g. The reference strains are also shown in Table S3 in the supplemental material.

Partial sequencing. Strains with double peaks and indicated with wobble IUPAC–International Union of Biochemistry (IUB) symbols were 06-5121 (stx_{2c} and stx_{2d}), B2F1 (stx_{2d1} and

stx_{2d2}), EBC287 (stx_{2b}, stx_{2c}, and stx_{2d}), E32511 (stx_{2a} and stx_{2c}), 031 (stx_{2b} and stx_{2c}), A397 (stx_{2a} and stx_{2c}), CL-3 (stx_{2a} and stx_{2d}), EBC275 (stx_{2b} and stx_{2d}), EC1720a (stx_{2b} and stx_{2d}), EC173b (stx_{2b} and stx_{2d}), I6581 (stx_{2a} and stx_{2c}), and VTB60 (stx_{2a} and stx_{2c}). Double peaks were not observed with any of the stx_1-positive strains.

Development and validation of the subtyping protocol. All 48 strains selected from the 62 reference collection strains for detection and subtyping were identified with the suggested subtyping primers at the expected fragment size except strain 7279 (D3279), which gave a band of approximately 2.1 kb in two laboratories. Toxin genes cloned from two strains with pEHEC400 and pVTEC9 (D3511 and D3514) were nontoxigenic and, therefore, were not subtyped by two laboratories.

Subtyping of stx_1 was correct for 30 of 48 strains at the initially prescribed annealing temperature of 62°C. Sixteen strains (five strains in two, five strains in one, three strains in one, two strains in one, and one strain in two laboratories) were correctly subtyped for stx_1 upon retesting at an annealing temperature of 64°C.

Subtyping of stx_2 was correct for 16 of 48 strains at the initially prescribed annealing temperature of 62°C. One laboratory (laboratory 1) returned correct results for 11 strains upon retesting at an annealing temperature at 64°C but had to run gradient PCR on an additional four strains and determined that false-positive results for stx_{2c} could be eliminated at an annealing temperature of 66°C. Laboratory 2 obtained correct results for seven strains upon retesting, laboratory 3 for 20 strains after changing to the HotStarTaq Master Mix Kit (Qiagen) at an annealing temperature at 64°C, laboratory 4 for eight strains, and laboratory 5 for six strains. Laboratories 2 and 4 were advised to retest two and four strains, respectively, for stx_{2c} at an annealing temperature at 66°C and finally obtained correct results.

Test of subtyping protocol on clinical isolates. Of the 162 clinical isolates, 40 strains were positive for stx_1, 45 for stx_2, 33 for stx_{2f}, and 44 for both stx_1 and stx_2 by dot blot hybridization. The following subtypes and combinations were detected using the subtyping protocol developed in this study: stx_{1a} (34 strains), stx_{1a} and stx_{2a} (6), stx_{1a} and stx_{2b} (3), stx_{1a} and stx_{2c} (8), stx_{1a} and stx_{2d} (1), stx_{1c} (6), stx_{1c} and stx_{2b} (11), stx_{1c} and stx_{2a}, and stx_{2b} (1), stx_{1d} (4), stx_{2a} (18), stx_{2a} and stx_{2c} (8), stx_{2a} and stx_{2d} (2), stx_{2b} (12), stx_{2b} and stx_{2c} and stx_{2d} (1), stx_{2b} and stx_{2d} (1), stx_{2c} (5), stx_{2d} (5), stx_{2e} (3), and stx_{2f} (33).

Forty-two strains from the German HUSEC collection (35) were tested with the subtyping protocol, and the results are listed in Table 3. Two Stx1-encoding strains were negative by the triplex PCR for stx_1. The strains were retested by classical typing as described in reference 66 and confirmed negative, indicating that loss of the stx_1 genes had occurred. HUSEC028 was originally typed as stx_{2d} but was positive for stx_{2b} by the subtyping protocol.

Activatability. Of the 23 strains tested for activatability, none of the 10 strains without the stx_{2d} gene were activatable as shown by VCA following treatment of culture supernatants with mouse mucus: strains 94CR, encoding Stx1a and Stx2a, A75, encoding Stx1a and Stx2c, DG131/3, encoding Stx1c and Stx2b, MHI813, encoding Stx1d, 126814, encoding Stx2a, VTB60, encoding Stx2a and Stx2c, EH250, encoding Stx2b, 031, encoding Stx2b and Stx2c, and 3615/99 and E-D53, encoding Stx2e. Mucus treatment of the supernatant from strain H.I.8. (which encodes Stx2f) showed a 4- to 5-fold activation on four occasions, but a 5-fold activation did not meet the threshold for activation. The supernatant from strain 7V, encoding Stx2g, averaged 5-fold activation, but the activation tests showed wide variations. A total of eight strains (EBC275, EC1720a, and EC173b, encoding Stx2b and Stx2d, EBC287, encoding Stx2b and Stx2c and Stx2d, and B2F1, EC1871a, F61029, and C165-02, encoding Stx2d) were all above the 6-fold activation threshold (range, 6- to 28-fold), and one strain, 06-5121, encoding Stx2c and Stx2d, was somewhat elevated (2- to 2.5-fold). We observed that pVTEC7, with the cloned Stx2d gene from strain EBC281, did not express toxin at all. We showed that strain MT71, previously published as stx_{2c} based on RFLP analysis (26), gained a PstI site, was activatable, was typed as stx_{2d} with our subtyping protocol, and fell into the Stx2d cluster by sequence analysis (Fig. 2 and 3).

DISCUSSION

We analyzed 398 toxin sequences (85 *stx*/stx_1 and 311 stx_2 plus two not specified) using a phylogenetic approach and identified a total of 285 valid (47 *stx*/stx_1 and 238 stx_2) sequences. Based on this analysis, and using the existing nomenclature as a backbone for our scheme, we developed a three-tiered nomenclature system that consists of three levels of designations for these cytotoxins: types, subtypes, and variants.

(i) Types. Types are the two major branches of the Shiga toxin family that share structure and function but that are not cross neutralized with heterologous antibodies (Stx/Stx1 and Stx2). For historical reasons, the Stx/*stx* nomenclature (no Arabic numbers) is reserved for Shiga toxin and the genes encoding it when they occur in *Shigella* spp. Designations for Shiga toxins or the genes encoding these toxins when they occur in *E. coli* and other bacteria include an Arabic number after "Stx" or "*stx*." Stx1 and Stx2 should only be used when the subtype is unknown.

(ii) Subtypes. The antigenically related members of the two main types, including Stx1 subtypes, are Stx (Shiga toxin from *Shigella* spp.) and the Shiga toxin subtypes found in *E. coli*, which are suffixed with small Arabic letters, Stx1a, Stx1c, and Stx1d. Stx2 is also suffixed with small Arabic letters, Stx2a to Stx2g, when they are from *Acinetobacter haemolyticus*, *Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia albertii* (45), and *Escherichia coli*.

(iii) Variants. Variants include the subtype-specific prototypic toxins or related toxins within a subtype (that differ by one or more amino acids from the prototype). The variants are designated by toxin subtype, O group if the host strain is *E. coli* and generic name of the host bacterium if the host strain is not *E. coli*, followed by the strain name or number from which that toxin was described. These determinants are separated by hyphens, as in Stx1a-O157-EDL933 or Stx2c-O157-E32511. Nucleotide variants within a given Stx subtype are italicized (e.g., stx_{2a}-O83-N1135 is a nucleotide variant that encodes Stx2a-O113-TS17-08). For identical sequences, the date of publication is given preference for choice of variant designation.

For reasons of simplicity and in order to minimize problems with database entries, only hyphens should be used for naming both amino acid and nucleotide variants, i.e., in species names and strain designations (e.g., *Acinetobacter haemolyticus* strain DS9B encodes Stx2a-Acinetobacter-haemolyticus-DS9B, strain T4/97 encodes Stx2f-O128-T4-97, strain H.I.8. encodes Stx2f-O89-HI8, etc.). In summary, the variant name includes the O serotype and strain name of the organism in which the toxin was detected. Toxin type 1 includes Stx and Stx1, but the prototypic Stx and Stx1 toxins were grouped within one new subtype, Stx/Stx1a. The other

TABLE 3 Results from use of the subtyping protocol developed in this study on the German HUSEC collection compared to the results obtained by classical typing as described in references 16, 17, and 66

HUSEC no.	Strain no.	Serotype	*stx* classical typing as described in references 16, 17, and 66	Subtyping protocol (this study)
HUSEC001	05-946	O111:H10	2	stx_{2a}
HUSEC002	5152/97	Ont:H-	2	stx_{2a}
HUSEC003	6334/96	O157:H7	2	stx_{2a}
HUSEC004	3072/96	O157:H-	2	stx_{2a}
HUSEC005	2907/97	O55:H7	2	stx_{2a}
HUSEC006	5376/99	O157:H-	2	stx_{2a}
HUSEC007	7382/96	O103:H2	2	stx_{2a}
HUSEC008	2791/97	O103:H-	2	stx_{2a}
HUSEC009	6833/96	OR:H2	2	stx_{2a}
HUSEC010	1805/00	O119:H2	1	stx_{1a}
HUSEC011	2516/00	O111:H8	1 and 2	stx_{1a} and stx_{2a}
HUSEC012	6037/96	O111:H-	1 and 2	stx_{1a} and stx_{2a}
HUSEC013	2245/98	O26:H11	1	stx_{1a}
HUSEC014	5080/97	O26:H-	1 and 2	stx_{1a} and stx_{2a}
HUSEC015	126814/98	OR:H11	1 and 2	stx_{2a} (stx_1 lost[a])
HUSEC016	5028/97	Ont:Hnt	1	stx_{1a}
HUSEC017	3319/99	O26:H11	1 and 2	stx_{1a} and stx_{2a}
HUSEC018	1530/99	O26:H11	2	stx_{2a}
HUSEC019	1588/98	OR:H11	1	stx_{1a}
HUSEC020	3271/00	O26:H11	2	stx_{2a}
HUSEC021	0488/99/A	O145:H28	2	stx_{2a}
HUSEC022	4557/99	O145:H-	2	stx_{2a}
HUSEC023	1169/97/1	O112:H-	2dact	stx_{2d}
HUSEC024	2996/96	O73:H18	2dact	stx_{2d}
HUSEC025	06-05009	O55:Hnt	1	stx_{1a}
HUSEC026	99-09355	O113:H21	2dact	stx_{2d}
HUSEC027	03-07727	O163:H19	2dact	stx_{2d}
HUSEC028	03-06687	O128:H2	1c and 2d	stx_{1c} and stx_{2b}
HUSEC029	4256/99	O70:H8	2	stx_{2a}
HUSEC030	05-03519	O98:H-	1	stx_{1a}
HUSEC031	7792/96	OR:H-	1	stx_{1a}
HUSEC032	2441/98	O136:Hnt	1c and 2	stx_{1c} and stx_{2a}
HUSEC033	4392/97	O145:H25	2	stx_{2a}
HUSEC034	3332/99	O91:H21	1 and 2 and 2dact	stx_{1a} and stx_{2a} and stx_{2d}
HUSEC035	1529/98	O121:H19	2	stx_{2a}
HUSEC036	2839/98	O145:H-	1 and 2c	stx_{2c} (stx_1 lost[a])
HUSEC037	02-03885	O104:H21	1 and 2 and 2dact	stx_{1a} and stx_{2a} and stx_{2d}
HUSEC038	3356/97B	Ont:H21	1 and 2dact	stx_{1a} and stx_{2d}
HUSEC039	3651/96	O76:H19	1c	stx_{1c}
HUSEC040	220/00	O174:H21	2c	stx_{2c}
HUSEC041	01-09591	O104:H4	2	stx_{2a}
HUSEC042	820/08	O165:H25	2 and 2c	stx_{2a} and stx_{2c}

[a] Loss of stx_1 was confirmed using classical typing as described in reference 66.

subtypes were those already described as Stx1c and Stx1d. Stx2 toxins were further defined with the addition of two new subtypes, Stx2a (the prototypic Stx2 sequence) and Stx2b (including the previously named VT2d variant), and the five existing subtypes, i.e., Stx2c, Stx2d (activation potential implied by sequence, see below), Stx2e, Stx2f, and Stx2g.

The sequence-based phylogenetic analyses included the intergenic regions and the identification of common motifs within each subtype and further supported the naming of subtypes. In particular, we hypothesized that two motifs in combination that are only present in variants of subtype Stx2d are related to the activatable property of this subtype. Subsequent testing for activation with intestinal mouse mucus confirmed this hypothesis in all nine strains within the Stx2d cluster, which contained these two motifs and were activatable by a factor of 6- to 28-fold. Only one strain, 06-5121, encoding Stx2c and Stx2d, did not meet the threshold for activatability. It is likely that the production of additional toxin Stx2c, which is more active on Vero cells than Stx2d, masked the activation phenotype in that strain.

The alignment of all known sequences also allowed us to evaluate some of the existing subtyping methods and identify theoretical pitfalls and possible misinterpretations of PCR-RFLP results. These methods have never really been validated against a representative number of strains. Bastian et al. (3) used only nine strains to validate 14 PCR systems and create a subtyping scheme. Piérard et al. (51) supplemented this with a method adding only one strain (EH250) to this panel. In a much more comprehensive study, Ziebell et al. (68) used two PCR protocols, nine subtyping

protocols, and three RFLP protocols on 12 reference strains and 496 field strains. They observed that the PCR-RFLP protocols gave contradictory results for approximately 20% of the strains tested and developed additional primers in order to allow for subtyping of all the studied subtypes and variants. None of these studies have used the same nomenclature for the toxins, nor have they addressed the problem of how to name the many variants using a systematic approach.

One of the cornerstones of RFLP typing has been the absence of the PstI site (position 908 to 913), which has been used as an indicator of the presence of the mucus-activatable stx_{2d} subtype (10, 14, 21, 26). However, the PstI site is also absent in 5 variants of stx_{2a} in *E. coli* (stx_{2a}-O113-CL-3, stx_{2a}-ONT-EBC217, stx_{2a}-O104-G5506, stx_{2a}-O8-VTB178, and stx_{2a}-ONT-pEHEC400) and in stx_{2a}-E-cloacae-95MV2, in two variants of stx_{2c} (stx_{2c}-O171-EBC287 and stx_{2c}-ONT-EBC289), in stx_{2f}, and in all four variants of subtype stx_{2g}. Using the protocol developed in this study, HUSEC028 strain serotype O128:H2, previously described as stx_{2d} by classical typing (16), was subtyped as stx_{2b} (Table 3). This can be explained by two point mutations within the PstI site (at position 909, T → A, and position 912, A → T). Similarly, strain MT71, encoding Stx2d, has acquired the PstI site, leading to misinterpretation as Stx2c. Thus, several variants within a given subtype may have single restriction enzyme (RE) site changes that would lead to misinterpretation by RFLP analysis. Furthermore, the primers often used for this RFLP typing method have been SLT-II-vc and CKS2, of which the latter is situated outside the locus of the stx_2 gene itself. The primers developed and tested by us during this study have all been designed to lie within the structural gene for the A and B subunits in order to ensure that all the analyzed sequences contained the matching sequences.

Our multicenter validation of the PCR typing protocol revealed several areas of potential variability in results. Because Stx2a, Stx2c, and Stx2d are very closely related, they posed a special challenge to the design of specific primers and determination of optimal stringency. We identified 30 stx_{2a}, 24 stx_{2c}, and 26 stx_{2d} nucleotide variants. The primers that we designed were discriminating of these variants in this study; however, other variants may exist that cannot be subtyped with these primers. Furthermore, we noted that cross-reactions occurred and appeared as ghost bands on gel electrophoresis, especially between stx_{2c}- and stx_{2d}-positive strains, so additional stringency may be needed to differentiate those toxin subtypes.

We observed differences in subtype results among the participating laboratories that we attributed to the use of different reagents and thermocyclers. A prototype protocol was adopted in an External Quality Assurance (EQA) scheme that involved both the networks of medical and veterinary/food National Reference Laboratories of the European Union (EU) member states and other laboratories outside the EU. The EQA was conducted jointly by the WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* in Copenhagen and by the EU Reference Laboratory for *E. coli* in Rome, Italy, and also aimed at the harmonization of the typing methods used in both the networks, to favor the comparison of data referring to human and nonhuman isolates of STEC. The study, funded by the European Centre for Disease Prevention and Control (ECDC) and by the European Commission, was conducted in 2011, included 70 participating laboratories (unpublished data), and indicated that the prototype PCR subtyping protocol was subject to variability based on the accuracy of annealing temperatures in thermocyclers, primer quality, and manufacturer of the polymerases. Independently, the participating laboratories indicated that they had unwanted cross-reactions when subtyping strains with stx_{2a}, stx_{2c}, and stx_{2d}. In our study, this problem was resolved by raising the annealing temperature to 64 to 66°C. Gradient testing by two of the participating centers in this study [Istituto Superiore di Sanità (ISS) and SSI] on different thermocyclers showed that the maximum annealing temperatures may vary a little in different laboratories. Nonetheless, with careful standardization and use of the set of control strains provided to each lab, we showed that correct results were achieved by the majority of reference laboratories. The revised and final protocol for subtyping the Stx genes adopted from this study specifying the necessity for individual calibration of annealing temperatures on different brands of thermocyclers is described in Table 1 and is available online (57).

Among the unresolved discrepancies were the following observations. Unexpected results for strain 7279 were obtained in two laboratories and negative in four laboratories. Strain 7279 was negative for toxin by VCA but positive by Ridascreen EIA (8). Our phylogenetic analysis placed this stx_2 variant in the stx_{2d} cluster, but subtyping was positive for stx_{2c}. These results may be explained by the possible insertion of an IS element in the toxin gene, as indicated by the presence of a fragment larger than expected by the stx_2 detection primers in two laboratories. The insertion of insertion sequence elements in the Stx-coding genes has been seen in other STEC O157 strains (31).

A number of phylogenetic analyses have been published over the past decade (2, 13, 32), but none of them provide the extensive sequence comparison shown here. Our phylogenetic trees correspond with trees in earlier publications (2, 3, 13, 32, 51) except for our inclusion of Stx2g and our naming of subtypes Stx1a, Stx2a, and Stx2b. To avoid confusion with the toxin subunits A and B (uppercase), we propose that subtype names always be in lowercase letters. Hence, the A subunit protein of Stx1a should be designated StxA1a, the B subunit protein of Stx2a should be designated StxB2a, etc. We propose that toxin operons or open reading frames be written as *stx* for holotoxin, *stxA* for the A subunit gene, and *stxB* for the B subunit gene in italics, with the type and subtype written as alleles without italics and in subscript. Thus, the gene encoding the A subunit of Stx1a should be written $stxA_{1a}$ and the B subunit gene of the same toxin as $stxB_{1a}$. In databases that do not allow the use of italics or subscripts, the first letter will define toxin (Stx) or gene (stx).

Universal typing schemes such as serotyping of *Salmonella* and *E. coli*, pulsed-field gel electrophoresis, and multiple-locus variable-number tandem repeat analysis have demonstrated their usefulness in epidemiology, risk assessment, and outbreak detection for several bacterial species. Similarly, a universal language for Stx taxonomy is essential for the comparison of STEC strains among research and public health laboratories and for the surveillance of STEC strains regionally, nationally, and internationally. Standard methods and nomenclature are also necessary to elucidate associations between toxin subtypes and specific clinical features and to assess the risks of STEC in populations and agricultural reservoirs. One such important observation within the past 6 to 7 years has been the association of Stx2a with *eae*-positive strains and Stx2d with *eae*-negative strains and their links with HUS. By defining a common nomenclature and an accompanying subtyping protocol, we hope to strengthen these studies to achieve a better under-

standing of these associations and trends and the risks to public health.

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